

(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization  
International Bureau



(43) International Publication Date  
1 August 2002 (01.08.2002)

PCT

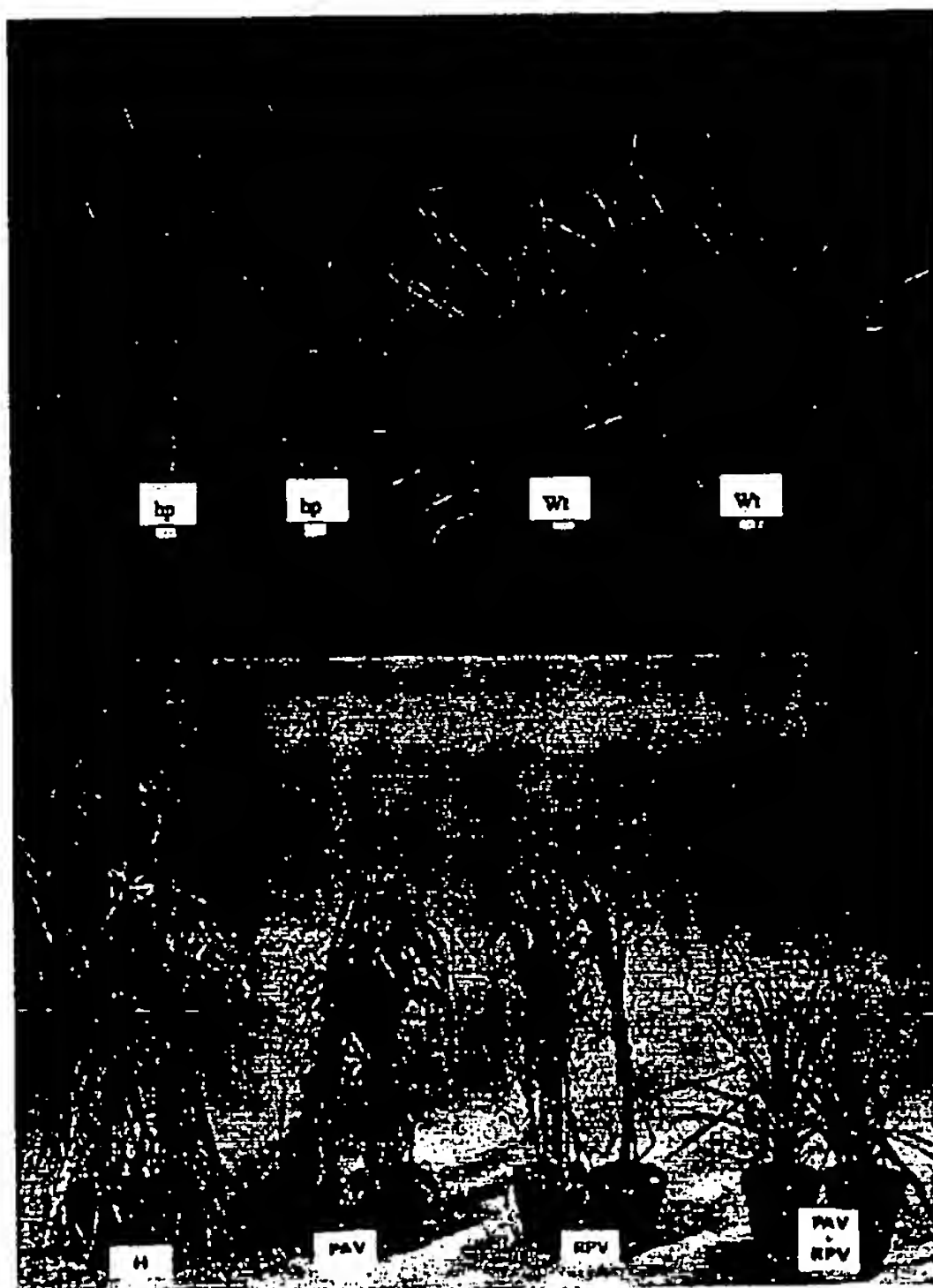
(10) International Publication Number  
**WO 02/059257 A2**

- (51) International Patent Classification<sup>7</sup>: C12N
- (21) International Application Number: PCT/IB01/02737
- (22) International Filing Date: 31 October 2001 (31.10.2001)
- (25) Filing Language: English
- (26) Publication Language: English
- (30) Priority Data:  
60/244,209 31 October 2000 (31.10.2000) US
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- (81) Designated States (*national*): AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DZ, EC, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NO, NZ, OM, PH, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZW.
- (84) Designated States (*regional*): ARIPO patent (GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZW), Eurasian

[Continued on next page]

(54) Title: METHOD AND MEANS FOR PRODUCING BARLEY YELLOW DWARF VIRUS RESISTANT CEREAL PLANTS

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B

(57) Abstract: The invention provides a DNA molecule comprising a plant-operable promoter operably linked to a DNA region that is capable of being transcribed in the cells of a cereal plant to produce RNA comprising inverted repeat sequence least about 19 nucleotides from the sequence of an RNA dependent RNA polymerase gene of Barley Yellow Dwarf Virus (BYDV). The DNA molecule is useful for reducing expression of the viral RNA dependant RNA polymerase and for enhancing the resistance of cereal crops to BYDV, optionally in the presence of a viral product that normally inactivates other modes of post transcriptional gene silencing. Methods are provided for producing transgenic cereal plant lines comprising the DNA molecule of the invention integrated into their genome, and selecting those lines having resistance to BYDV infection. Transgenic plants having enhanced resistance to BYDV are also provided.

WO 02/059257 A2



patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE, TR), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

*For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.*

**Published:**

- *without international search report and to be republished upon receipt of that report*

**Methods and means for producing Barley Yellow Dwarf virus resistant  
cereal plants.**

**5 Field of the invention.**

The invention relates to methods for producing transgenic cereal plants resistant to Barley Yellow Dwarf Virus, particularly in the presence of co-infecting Cereal Yellow Dwarf Virus, by stably integrating into the cells of the transgenic plant a  
10 chimeric gene comprising a DNA region operably linked to plant expressible promoter in such a way that a RNA molecule may be transcribed from the DNA region, the RNA molecule comprising both sense and antisense RNA capable of pairing and forming a double stranded RNA molecule or hairpin RNA.

**15 Description of related art**

Barley yellow dwarf virus–PAV (BYDV-PAV) is the most serious and widespread virus of cereals worldwide. The barley yellow dwarf virus (BYDV), also called red leaf in oats, can infect barley, oats, rye and wheat as well as numerous species  
20 of grasses. It occurs in most parts of the world and is considered the most common viral disease of cereal crops.

BYDV (and CYDV) have been reported to cause cereal disease in over 50 countries. In almost all cases where the species has been determined, the major  
25 losses have been due to BYDV-PAV (Barker & Waterhouse, 1999). Lost production due to BYDV averages about 15% in barley, 17% in wheat and 25% in oats (Lister and Ranieri, 1995). BYDV infection can affect plant height, grain size and grain quality. For example, BYDV infection of barley can reduce the grain quality such that it is suitable only for animal feed rather than malting.

BYDV is transmitted by several species of aphids. As the aphids (winged or wingless) feed on the cereal crop, they transmit the virus through their mouthparts. The aphids can remain infectious for life, which is around 40 days.

- 5 Disease symptoms vary with the host species and the stage of crop development. Infections at the seedling stage may result in death or dwarfing as well as sterile heads. Leaves turn yellow from the tip down, along the leaf margins or in blotchy patches. Infected barley leaves, particularly flag leaves, turn bright yellow. In oats, the leaves may turn from red to purple. Discolored areas  
10 enlarge and progress to the base of the plant. Heads may be wholly or partially sterile. There may also be an increase or decrease of tillers produced by infected plants. Cereal plants infected early in the season may be shaded out by healthy or late infected surrounding plants. Winter wheat seedlings may be 100 per cent infected with BYDV before freeze-up in the fall. BYDV affects yields by stunting,  
15 reduced tillering, sterility, and failure to fill kernels.

Natural resistance genes against this luteovirus give inadequate control. Sources of natural resistance to BYDV and CYDV are rare (for reviews see Barker and Waterhouse 1999; Burnett *et al.*, 1995). In barley, the *Yd2* gene (Paltridge *et al.*,  
20 1998), originally identified in Ethiopian concessions (Rasmussin and Schaller, 1959; Schaller *et al.*, 1964), can confer resistance against BYDV-PAV, but its effectiveness varies depending on the genetic background of the plant and growth conditions (Schaller, 1984; Larkin *et al.*, 1991). The *Bdv1* gene confers some tolerance to BYDV (Singh *et al.*, 1993) and has been introduced into some  
25 wheat cultivars, such as Anza. However, BYDV replicates and causes symptoms and yield loss in plants containing either the *Yd2* or *Bdv1* genes.

Previous attempts to introduce synthetic resistance into cereals have produced variable results. Virus resistance in plants containing virus-derived transgenes,  
30 usually by the expression of functional or dysfunctional coat protein, movement or polymerase genes, has been widely reported (for review see Waterhouse &

Upadhyaya 1998) and attempts have been made to produce transgenic plants with resistance to a few different luteoviruses, by expression of coat protein and polymerase genes.

- 5 McGrath *et al.* (1997) transformed oat and barley plants with transgenes derived from the coat protein genes of BYDV-PAV and CYDV-RPV and obtained some resistant plants. However, this resistance was not stable.

- 10 In another study, Koev *et al.* (1998) transformed oat plants with the 5' half of the BYDV-PAV genome and found one line that after inoculation with BYDV-PAV showed disease symptoms but recovered and produced seed. Although BYDV resistance in the progeny of this line was inherited, the levels of resistance varied greatly among individual plants, ranging from substantial to undetectable.

- 15 Waterhouse *et al.*, 1998, Wang & Waterhouse, 2000 and Smith *et al.*, 2000 describe that virus immunity and posttranscriptional gene silencing (PTGS) can be induced in plants using transgenes that encode double stranded (ds) or self-complementary "hairpin" (hp) RNA.

- 20 WO 98/53083 describes constructs and methods for enhancing the inhibition of a target gene within an organism involve inserting into the gene-silencing vector an inverted repeat sequence for all or part of a polynucleotide region within the vector. The inverted repeat sequence may be a synthetic polynucleotide sequence or comprise a modified natural phenotype.

- 25 WO 99/32619 provides a process of introducing RNA into a living cell to inhibit gene expression of a target gene in that cell. The RNA has a region with a double-stranded structure. Inhibition is sequence-specific in that the nucleotide sequence of the duplex region of the RNA and of a portion of the target gene are  
30 identical.

WO 99/49029 relates to a method of modifying gene expression and to synthetic genes for modifying endogenous gene expression in a cell, tissue or organ of a transgenic organism, in particular a transgenic animal or plant. Recombinant DNA technology is used to post-transcriptionally modify or modulate the expression of a target gene in a cell, tissue, organ or whole organism, thereby producing novel phenotypes. Synthetic genes and genetic constructs which are capable of repressing, delaying or otherwise reducing the expression of an endogenous gene or a target gene in an organism when introduced thereto are also provided.

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WO 99/53050 provides methods and means for reducing the phenotypic expression of a nucleic acid of interest in eucaryotic cells, particularly in plant cells, by introducing chimeric genes encoding sense and antisense RNA molecules directed towards the target nucleic acid, which are capable of forming a double stranded RNA region by base-pairing between the regions with sense and antisense nucleotide sequence or by introducing the RNA molecules themselves. Preferably, the RNA molecules comprise simultaneously both sense and antisense nucleotide sequence.

WO 99/61631 relates to methods to alter the expression of a target gene in a plant using sense and antisense RNA fragments of the gene. The sense and antisense RNA fragments are capable of pairing and forming a double-stranded RNA molecule, thereby altering the expression of the gene. This publication also relates to plants, their progeny and seeds derived thereof, obtained using the methods described.

WO 00/49035 discloses a method for silencing the expression of an endogenous gene in a cell, the method involving overexpressing in the cell a nucleic acid molecule of the endogenous gene, wherein the overexpression of the nucleic acid molecule of the endogenous gene and the antisense molecule in the cell silences the expression of the endogenous gene.



## SUMMARY AND OBJECTS OF THE INVENTION

The invention provides DNA molecules comprising a plant-expressible promoter operably linked to a DNA region which when transcribed in the cells of a cereal plant yields an RNA molecule comprising a first nucleotide sequence of at least 19bp having at least 70% nucleotide sequence identity to the sense nucleotide sequence of a BYDV isolate encoding the polymerase gene and a second nucleotide sequence of at least 19bp having at least 70% nucleotide sequence identity to the complement of the sense nucleotide sequence of an RNA dependent RNA polymerase (hereinafter referred to as "polymerase") gene of a BYDV isolate, such as but not limited to a polymerase comprising the nucleotide sequence of SEQ ID No 1 from the nucleotide at position 1 to the nucleotide at position 1014, and optionally a transcription termination and polyadenylation signal, wherein the first and second nucleotide sequence are capable of forming a double stranded RNA by base pairing between regions which are complementary. The first nucleotide sequence may comprise the nucleotide sequence of SEQ ID No 1 and the second nucleotide sequence may comprise the complement of the nucleotide sequence of SEQ ID No 1. The DNA molecules of the invention may contain a nucleotide sequence encoding a spacer region between the DNA region encoding the first nucleotide sequence and the DNA region encoding the second nucleotide sequence. The spacer region may have the nucleotide sequence of SEQ ID No 2.

The invention also provides a method for producing a cereal plant, such as a cereal plant selected from the group of wheat, barley, rye and oats, e.g. barley, resistant to a Barley Yellow Dwarf Virus comprising the steps of producing a population of transgenic cereal plant lines comprising the DNA molecules of the invention integrated into the genome of the cells of transgenic plant of the plant line and selecting a transgenic cereal plant line resistant to Barley Yellow Dwarf virus infection and optionally comprising the further step of crossing the selected

transgenic cereal plant line resistant to Barley Yellow Dwarf virus infection to another cereal plant to obtain progeny plants comprising the DNA molecules of the invention.

- 5 Another objective of the invention is to provide a method for producing a cereal plant resistant to a Barley Yellow Dwarf Virus in the presence of a post transcriptional gene silencing inactivating protein, which may be encoded by a co-infecting virus, particularly Cereal Yellow Dwarf Virus, comprising the step of producing a population of transgenic cereal plant lines comprising a DNA
- 10 molecule of the invention integrated into the genome of the cells of transgenic plant of said plant line and selecting transgenic cereal plant lines resistant to Barley Yellow Dwarf Virus infection.

- Yet another objective of the invention is to provide use of a DNA molecule of the
- 15 invention to produce a transgenic cereal plant resistant to BYDV.

- It is also a further objective of the invention to provide the use of a DNA molecule of the invention to produce a transgenic cereal plant resistant to BYDV in the presence of Cereal Yellow Dwarf virus (CYDV).

20

- The invention further provides cereal plants and plant lines, which may be selected from the group consisting of wheat, barley, rye and oat, e.g. barley plant and plant lines comprising stably integrated into the genome of the cells of the cereal plant a DNA molecule according to the invention wherein the cereal plant
- 25 is resistant to BYDV virus infection and replication of said virus, also in the presence of CYDV.



## BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1. (A) Genome map of BYDV-PAV showing regions used to generate hpBYDVpol. (B) Design of hpBYDVpol construct. (C) Diagram of self-complementary (hairpin) RNA produced by hpBYDVpol. RB: right border; nos 3': nopaline synthase 3' region; 35S-P: cauliflower mosaic virus 35S promoter; tm1': tumour morphology large gene 3' region; PAVpol: BYDV-PAV polymerase gene sequence; JGMV5': Johnson grass mosaic virus 5'untranslated region; Ubi-P: maize polyubiquitin gene promoter; LB: left border.

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Figure 2. Southern blot analysis of 15 primary T<sub>0</sub> hpBYDVpol barley transformants. DNA was digested with *Hind* III, separated by electrophoresis, blotted to Hybond N+™ membrane and hybridized with a radioactively labelled *hpt* probe. The number of intense bands in each in each lane should represent the number of transgene copies in the plant line. Weak bands may be due to partial digestion of DNA.

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Figure 3. Southern blot analysis of T<sub>1</sub> progeny of hpBYDVpol Lines 2 and 4. DNA from two resistant (R) and one susceptible (S) T<sub>1</sub> plants for Lines 2 and 4, and for non-transgenic barley (C), was digested with *Apa* I or *Bam*H I, separated by electrophoresis, blotted to Hybond N+™ membrane and hybridized with a radioactively-labelled *hpt* probe. The number of bands in the *Apa* I lanes and the intensity of the 3.75 KB bands for the *Bam*H I lanes should indicate copy number.

20

Figure 4. Relationship between virion accumulation and inheritance of the hpBYDVpol transgene. Virus levels detected by ELISA 21 (white bar), 28 (grey bar) and 42 (black bar) days after inoculation in (A) 14 T<sub>1</sub> progeny of Line 2 and (B) 12 T<sub>1</sub> progeny of Line 4. Displayed above each histogram is a symbol representing the severity of viral symptoms in mature plants and an agarose gel containing PCR products from the corresponding plant samples; the presence of

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a 626bp product indicates the amplification and detection of the hpBYDVpol transgene.

Figure 5. Reaction of transgenic barley, non-transgenic barley, and oats to BYDV and CYDV. A. Reaction of transgenic, and non-transgenic barley plants to BYDV-PAV. Two T1 hpBYDVpol resistant plants of Line 2 (left) and two non-transformed barley plants (right). B. Coast-black oats (*Avena sativa*) infected with BYDV-PAV, CYDV-RPV and B/CYDV-MIX, a mixture of both isolates.

Figure 6. BYDV-PAV and CYDV-RPV accumulation in a population of segregating progeny from hpBYDVpol Line 2. Virus levels, detected by strain-specific ELISA, 21 days after inoculation, in 15 progeny plants inoculated with B/CYDV-MIX (a complex of both BYDV-PAV and CYDV-RPV). The pair of dark and light histogram bars for each progeny plant represent the levels of BYDV-PAV and CYDV-RPV detected, respectively. The + and - above each pair indicate whether or not the plant has inherited the hpBYDVpol transgene. The transgene status was determined by PCR as in Figure 3. C+ and C- are results for non-transgenic control plants that have been challenged and not challenged with B/CYDV-MIX, respectively.

## DETAILED DESCRIPTION OF PREFERRED EMBODIMENTS

Luteoviruses, such as Barley Yellow Dwarf Virus ("BYDV") have been among the most recalcitrant viral group to transgene-mediated resistance (Barker and Waterhouse, 1999). Despite the economic importance of the cereal diseases caused by BYDV, previous attempts to produce transgenic cereals with protection against BYDV have been unsuccessful.

While some attempts (McGrath et al., 1997; Koev et al., 1998) have produced oat or barley plants with resistance (reduced virus replication) or tolerance (reduced virus symptoms but unimpeded virus replication) to BYDV, the inheritance of the

resistance/tolerance has been variable. This inheritance has been further complicated by the complex transgene insertion patterns in such plants, especially those obtained using biolistic transformation.

- 5 The prior art is thus deficient in providing methods and means for obtaining BYDV resistant cereal plants, with stable inheritance of the resistance gene, using a transgenic approach. The current invention has solved these and other problems as set forth hereinafter in the various embodiments, as well as in the claims.

10

The current invention is based on the finding that transgenic cereal plants, such as barley, could be made extremely resistant to BYDV infection, replication and disease symptom development using a chimeric gene comprising a promoter which could be expressed in cereal plant cell, and could drive the transcription of  
15 an operably linked DNA region, the transcription resulting in an RNA molecule comprising both sense and antisense parts of a BYDV genome corresponding to the nucleotide sequence encoding the RNA dependent RNA polymerase (ORF1). The sense and antisense parts are essentially complementary to each other and thus capable of forming a double stranded RNA. For the virus resistance to occur  
20 such a chimeric gene need only to be present as a single transgene, thus resulting in inheritance in a simple Mendelian manner.

In addition, it was realized that the BYDV disease resistance conferred by the chimeric genes according to the invention, could be observed even in the  
25 presence of PTGS inactivating protein, such as may be found in the coinfecting Cereal Yellow Dwarf Virus (CYDV-RPV).

Until recently, BYDV was described as having at least six different serotypes, which fell into two different subgroups (Waterhouse *et al.*, 1988; Martin and  
30 D'Arcy, 1995). However, comparison of their nucleotide sequences has led to redefining PAV and MAV as species of BYDV, and RPV as a species of *Cereal*

*yellow dwarf virus* (CYDV; Mayo & D'Arcy 1999; Wang *et al.*, 1998). The 3' halves of the two viruses encode movement and coat protein genes and share a fair degree of homology. Their 5' halves encode polymerase gene sequences, which are more closely related to those of other groups of viruses than to each other (Miller *et al.*, 1995; Wang *et al.*, 1998a).

In one embodiment of the invention, a method is provided for producing a cereal plant or plant line resistant to Barley Yellow Dwarf virus infection, virus replication and disease symptom development comprising the steps of

- 10 a) producing a population of transgenic cereal plant lines comprising a DNA molecule integrated into the genome of the cells of a transgenic plant whereby the DNA molecule comprises the following operably linked elements:
  - (1) a plant-expressible promoter;
  - 15 (2) a DNA region which when transcribed in the cells of a cereal plant yields a RNA molecule comprising
    - (a) a first nucleotide sequence of at least 19 bp having at least 70% nucleotide sequence identity to the sense nucleotide sequence of a BYDV isolate encoding RNA dependent RNA polymerase;
    - 20 and
    - (b) a second nucleotide sequence of at least 19 bp having at least 70% nucleotide sequence identity to the complement of said sense nucleotide sequence of a BYDV isolate encoding RNA dependent RNA polymerase; and optionally
    - 25 (c) a transcription termination and polyadenylation signal;wherein the first and second nucleotide sequence are capable of forming a double stranded RNA by base pairing between regions which are complementary; and
- 30 b) isolating transgenic cereal plants or plant lines resistant to Barley Yellow Dwarf Virus infection.

For the purpose of this invention, the "sequence identity" of two related  
5 nucleotide or amino acid sequences, expressed as a percentage, refers to the  
number of positions in the two optimally aligned sequences which have identical  
residues (x100) divided by the number of positions compared. A gap, i.e. a  
position in an alignment where a residue is present in one sequence but not in  
the other is regarded as a position with non-identical residues. The alignment of  
10 the two sequences is performed by the Needleman and Wunsch algorithm  
(Needleman and Wunsch 1970) The computer-assisted sequence alignment  
above, can be conveniently performed using standard software program such as  
GAP which is part of the Wisconsin Package Version 10.1 (Genetics Computer  
Group, Madison, Wisconsin, USA) using the default scoring matrix with a gap  
15 creation penalty of 50 and a gap extension penalty of 3.

For the purpose of the invention, the "complement of a nucleotide sequence  
represented in SEQ ID No: X" is the nucleotide sequence which would be  
capable of forming a double stranded DNA molecule with the represented  
20 nucleotide sequence, and which can be derived from the represented nucleotide  
sequence by replacing the nucleotides through their complementary nucleotide  
according to Chargaff's rules ( $A \leftrightarrow T$ ;  $G \leftrightarrow C$ ) and reading in the 5' to 3' direction,  
i.e. in opposite direction of the represented nucleotide sequence.

25 As used herein, nucleotide sequences of RNA molecules may be identified by  
reference to DNA nucleotide sequences of the sequence listing. However, the  
person skilled in the art will understand whether RNA or DNA is meant depending  
on the context. Furthermore, the nucleotide sequence is identical except that the  
T -base is replaced by uracil (U) in RNA molecules.

The length of the first and second nucleotide sequences may vary from about 10 nucleotides (nt) up to a length equaling the length in nucleotides of part of the BYDV genome encompassing the RNA dependent RNA polymerase encoding ORF of a BYDV genome (normally referred to as ORF 1). The length of the first  
5 or second nucleotide sequence may be at least 15 nt, or at least about 20 nt, or at least about 50 nt, or at least about 100 nt, or at least about 150 nt, or at least about 200 nt, or at least about 500 nt, or at least about 1600 bp.

It will be appreciated that the longer the total length of the first nucleotide  
10 sequence is, the less stringent the requirements for sequence identity between the total sense nucleotide sequence and the corresponding sequence in the target gene become. The total first nucleotide sequence can have a sequence identity of at least about 75% with the corresponding target sequence, but higher sequence identity can also be used such as at least about 80 %, at least about  
15 85%, at least about 90%, at least about 95%, about 100%. The first nucleotide sequence can also be identical to the corresponding part of BYDV genome. However, it is advised that the first nucleotide sequence always includes a sequence of about 19 or 20 consecutive nt, or even of about 50 consecutive nt, or about consecutive 100 nt, or about 150 consecutive nt with 100% sequence  
20 identity to the corresponding part of BYDV genome. For calculating the sequence identity and designing the corresponding first nucleotide sequence, the number of gaps should be minimized, particularly for the shorter sense sequences.

25 The length of the second (antisense) nucleotide sequence is largely determined by the length of the first (sense) nucleotide sequence, and may correspond to the length of the latter sequence. However, it is possible to use an antisense sequence that differs in length by about 10% without any difficulties. Similarly, the nucleotide sequence of the antisense region is largely determined by the  
30 nucleotide sequence of the sense region, and may be identical to the complement of the nucleotide sequence of the sense region. Particularly with



longer antisense regions, it is however possible to use antisense sequences with lower sequence identity to the complement of the sense nucleotide sequence, such as at least about 75% sequence identity, or least about 80%, or at least about 85%, more particularly with at least about 90% sequence identity, or at least about 95% sequence to the complement of the sense nucleotide sequence. Nevertheless, it is advised that the antisense nucleotide sequences always includes a sequence of about 19 or 20 consecutive nucleotides, although longer stretches of consecutive nucleotides such as about 50 nt, or about 100 nt, or about 150 nt with 100% sequence identity to the complement of a corresponding part of the sense nucleotide sequence may be used. It is clear that the length of the stretch of the consecutive nucleotides with 100% sequence identity to the complement of the sense nucleotide sequence cannot be longer than the sense nucleotide sequence itself. Again, the number of gaps should be minimized, particularly for the shorter antisense sequences. Further, it is also advised that the antisense sequence has between about 75% to 100% sequence identity with the complement of the 5' half of the BYDV genome.

In one embodiment of the invention, the DNA molecules according to the invention may comprise a DNA region encoding a spacer between the DNA region encoding the first and second nucleotide sequences. As indicated in WO 99/53050 the spacer may contain an intron to enhance the BYDV resistance. In one embodiment thereof, the spacer may comprise the nucleotide sequence of SEQ ID No 2.

In another embodiment of the invention, the used DNA molecule has a first and second nucleotide sequence comprising a nucleotide sequence of at least 19 or 20 bp having at least 75 % sequence identity to a nucleotide region of a BYDV genome encompassing an ORF comprising the nucleotide sequence of SEQ ID No 1 from the nucleotide at position 1 to the nucleotide at position 1014, or its complement. It goes without saying that again the sequence identity may be higher, as indicated elsewhere in the specification or the length of the comprised

nucleotide sequence may be higher, again as indicated elsewhere in this specification.

In another embodiment of the invention, the first nucleotide sequence comprises  
5 the nucleotide sequence of SEQ ID No 1 and the second nucleotide sequence comprises the nucleotide sequence of the complement of SEQ ID No 1.

However, the nucleotide sequence of the complete genome of a number of  
BYDV isolates is known and available through databases such as e.g. Genbank  
10 and the person skilled in the art can easily identify the nucleotide sequences corresponding to the sequence of SEQ ID No1 in those nucleotide sequences. These nucleotide sequences derived from other BYDV isolates can be used to generate BYDV resistant cereal plants and plant lines to equal effect.

15 The following nucleotide sequences of complete genomes of BYDV isolates are available: Genbank accession Nr NC\_002160/ GI:9634102 ; Genbank accession Nr NC\_001599/ GI:9627413; Genbank accession Nr AF235167/ GI:7417288; Genbank accession Nr AF218798/ GI:6715477; Genbank Accession Nr D85783/ GI:1395150; Genbank Accession Nr D11032/ GI:221098; Genbank Accession Nr  
20 D10206/ GI:221091; Genbank Accession Nr D11028/ GI:221084; Genbank Accession Nr X07653/ GI:58798; Genbank Accession Nr L25299/ GI:408929.

Sequences corresponding to the nucleotide sequence used to exemplify the current invention may be identified in other BYDV genomes e.g. by using  
25 computer-assisted alignment programs. Alternatively, the sequences can be identified and isolated using PCR and the oligonucleotide primer with nucleotide sequences as in SEQ ID 3 and 4, essentially similar nucleotide sequences (having at least 85 % sequence identity, or at least 90% sequence identity).

30 It will be clear that also nucleotide molecules hybridizing under stringent conditions to a nucleotide comprising the sequence of SEQ ID No 1 or similar

corresponding sequences from other BYDV genomes may also be used to similar effect.

“Stringent hybridization conditions” as used herein mean that hybridization will  
5 generally occur if there is at least 95% and preferably at least 97% sequence identity between the probe and the target sequence. Examples of stringent hybridization conditions are overnight incubation in a solution comprising 50% formamide, 5 x SSC (150 mM NaCl, 15 mM trisodium citrate), 50 mM sodium phosphate (pH 7.6), 5x Denhardt’s solution, 10% dextran sulfate, and 20 µg/ml  
10 denatured, sheared carrier DNA such as salmon sperm DNA, followed by washing the hybridization support in 0.1 x SSC at approximately 65 °C. Other hybridization and wash conditions are well known and are exemplified in Sambrook et al, Molecular Cloning: A Laboratory Manual, Second Edition, Cold Spring Harbor, NY (1989), particularly chapter 11.

15

The invention is also directed to a method of protecting a cereal plant against BYDV virus in the presence of a PTGS inactivating protein. Such proteins and nucleotide regions encoding them have been identified in several viruses and endogenous orthologous genes in plants have been identified. As used herein, a  
20 PTGS inactivating protein is a protein, which when present in a plant cell results in a reduction of the post transcriptional gene silencing observed when sense or antisense RNA molecules are present in that cell.

In one embodiment, the PTGS inactivating protein is encoded by a virus, such as  
25 Cereal Yellow Dwarf Virus. The methods of the invention therefor can also be used to produce cereal plants resistant to Barley Yellow Dwarf Virus, and Cereal Yellow Dwarf Virus co-infection.

It goes without saying that the DNA molecules for use in the invention may  
30 comprise a first nucleotide sequence comprising several different sense nucleotide sequences corresponding to parts of a BYDV genome (as

hereinbefore described) and a second nucleotide sequence comprising the several corresponding complementary nucleotide sequences of the several different sense nucleotide sequences in the first nucleotide sequence. Moreover, a single DNA molecule for use in the invention may comprise within its first  
5 nucleotide sequence simultaneously several different sense nucleotide sequences corresponding to parts of a BYDV genome as well as different sense nucleotide sequences corresponding to parts of another viral genome such as a CYDV genome.

10 Thus, the invention also provides a method for protecting cereal plants, such as but not limited to barley plants, against a BYDV and CYDV co-infection, the method comprising

a) producing a population of transgenic cereal plant lines comprising a DNA molecule, wherein the DNA molecule comprises the following operably linked

15 DNA elements

i) a plant-expressible promoter;

ii) a DNA region which when transcribed in the cells of a cereal plant yields an RNA molecule comprising

20 1) a first nucleotide sequence comprising a multitude of nucleotide sequences, each comprising at least 19 or 20 bp having at least 70% nucleotide sequence identity to the sense nucleotide sequence of a BYDV isolate or a CYDV isolate

25 2) a second nucleotide sequence having at least 70 % nucleotide sequence identity to the complement of the first nucleotide sequence; and

iii) a transcription termination and polyadenylation signal; wherein the first and second nucleotide sequence are capable of forming a double stranded RNA by basepairing between the regions which are complementary; and

30 b) isolating transgenic cereal plant lines resistant to Barley Yellow Dwarf Virus and Cereal Yellow Dwarf Virus infection.

In one embodiment of the above mentioned method, the first nucleotide sequence comprises the nucleotide sequence of SEQ ID No 7 from the nucleotide at position 429 to the nucleotide at position 629 (corresponding to ORF1 of BYDV Australian isolate with the genomic sequence available under Genbank Accession number X 07653) and the nucleotide sequence of SEQ ID No 7 from the nucleotide at position 2314 to the nucleotide at position 2514 (corresponding to ORF2 of an Australian BYDV isolate) and the nucleotide sequence of SEQ ID No 7 from the nucleotide at position 5052 to the nucleotide at position 5270 (ORF6 +3' untranslated end of BYDV) and the nucleotide sequence of SEQ ID No 8 and the nucleotide sequence of SEQ ID No 9 (the latter two sequences corresponding to part of a CYDV genome) and the second nucleotide sequence comprises the about exact complement of the first nucleotide sequence.

15

The invention also extends to DNA molecules for use to produce BYDV resistant cereal plants described herein as well as plant cells and cereal plants comprising a DNA molecule according to the invention.

20 The DNA molecules of the invention suitable for the production of BYDV or BYDV/CYDV resistant plants may be introduced into cereal plant lines, already comprising the natural BYDV resistance alleles such as the mentioned *Yd2* or *Bdv1* genes to obtain an additive or synergistic effect on virus resistance.

25 The method and means of the invention are suited for cereal crop plants such as wheat, rye, oat and barley, but may also be used for protecting other grasses and small grain cereals susceptible to BYDV or closely related viruses.

It will be clear that actual method of transforming the cereal crop has little importance for the current method and several methods including *Agrobacterium* mediated transformation, microprojectile bombardment, electroporation of

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compact embryogenic calli, silicon whisker mediated DNA introduction are available in the art.

The obtained transformed plant can be used in a conventional breeding scheme to produce more transformed plants with the same characteristics or to introduce the DNA molecule for obtaining BYDV resistance according to the invention in other varieties of the same or related plant species, or in hybrid plants. Seeds obtained from the transformed plants contain the chimeric genes of the invention as a stable genomic insert and are also encompassed by the invention.

The following non-limiting Examples describe the construction of DNA molecules suitable for use in making BYDV resistant cereal plant lines. Unless stated otherwise in the Examples, all recombinant DNA techniques are carried out according to standard protocols as described in Sambrook and Russell (2001) *Molecular Cloning: A Laboratory Manual*, Third Edition, Cold Spring Harbor Laboratory Press, NY, in Volumes 1 and 2 of Ausubel et al. (1994) *Current Protocols in Molecular Biology*, Current Protocols, USA and in Volumes I and II of Brown (1998) *Molecular Biology LabFax*, Second Edition, Academic Press (UK). Standard materials and methods for plant molecular work are described in *Plant Molecular Biology Labfax* (1993) by R.D.D. Croy, jointly published by BIOS Scientific Publications Ltd (UK) and Blackwell Scientific Publications, UK. Standard materials and methods for polymerase chain reactions can be found in Dieffenbach and Dveksler (1995) *PCR Primer: A Laboratory Manual*, Cold Spring Harbor Laboratory Press, and in McPherson et al. (2000) *PCR - Basics: From Background to Bench*, First Edition, Springer Verlag, Germany.

All the nucleotide sequences identified in this text by their database accession number are hereby incorporated by reference.

Throughout the description and Examples, reference is made to the following sequences:



SEQ ID No 1: part of the Barley Yellow Dwarf Virus genome spanning ORF1 and  
5' end of ORF2.

5 SEQ ID No 2: part of the Barley Yellow Dwarf Virus genome spanning the 3' end  
of ORF2.

SEQ ID No 3: PCR oligonucleotide primer 1

SEQ ID No 4: PCR oligonucleotide primer 2

SEQ ID No 5: PCR oligonucleotide primer 3

SEQ ID No 6: PCR oligonucleotide primer 4

10 SEQ ID No 7: nucleotide sequence of an Australian isolate of BYDV (Genbank  
accession X07653)

SEQ ID No 8: nucleotide sequence of part of an Australian isolate of CYDV (from  
Genbank accession AF 020090)

15 SEQ ID No 9: nucleotide sequence of part of an Australian isolate of CYDV (from  
Genbank accession AF 020090)

## EXAMPLES

### Experimental procedures

#### 5 *Barley transformation*

*Hordeum vulgare* L. cv. 'Golden Promise' was transformed with the hpBYDVpol construct using the *Agrobacterium* inoculation procedure and target tissue as described by Tingay *et al.* (1997). Freshly dissected scutella (with embryo axes removed) were immersed for 10 min in a suspension of *Agrobacterium* 10 *tumefaciens* AGL1:hpBYDVpol or AGL0:hpBYDVpol, that was obtained by inoculating 5 mL of antibiotic-free MG/L medium (Tingay *et al.*, 1997) with 250 µL of overnight *Agrobacterium* culture (grown in the presence of 50 mg/L spectinomycin and 25 mg/L rifampicin) with shaking at 28°C. The scutella were blotted briefly with sterile filter paper to remove excessive liquid, placed with the 15 smooth side down on solid callus induction medium, and incubated at 25°C for 2-3 days. The scutella were then transferred without washing to callus induction medium containing 30 mg/L hygromycin and 150 mg/L Timentin™, again with the smooth side down. They were subcultured twice, at two weeks intervals, on callus induction medium containing 50 mg/L hygromycin and 150 mg/L 20 Timentin™ to produce hygromycin-resistant calli, which were subsequently transferred to regeneration medium containing 25 mg/L hygromycin and 150 mg/L Timentin™. Callus pieces forming shoots on the regeneration medium were transferred either directly to rooting medium containing 25 mg/L hygromycin or first to a half-strength regeneration medium and then to rooting medium.

25

#### *Testing transgenic plants for BYDV resistance*

Unless stated otherwise, aphids (*Rhopalosiphum padi*) were allowed to feed for 48 hr on virus-infected oat leaves then transferred (5-10 per plant) onto ten-day-old test plants. The virus isolates used were an Australian isolate of BYDV-PAV, 30 an Australian isolate of CYDV-RPV and an Australian B/CYDV-MIX isolate which contained both BYDV-PAV and CYDV-RPV (Waterhouse *et al.*, 1986). After a 72

hr inoculation feeding period on the test plants, the aphids were killed with pyrethrin and the plants transferred to a glasshouse (16 hr day 18°C; 8 hr night 13°C). Virion accumulation was measured, unless stated otherwise, 21 and 28 days after inoculation, by enzyme-linked immunosorbent assay (ELISA) (Xin *et al.*, 1988), using monoclonal antibody diagnostic kits for BYDV-PAV and CYDV-RPV supplied by Sanofi Pasteur Diagnostics. The conversion of substrate was measured after four hours incubation.

#### *Analysis of transgenic plants by DNA hybridization*

10 Genomic DNA was isolated from barley leaves using the procedure of Lagudah *et al.* (1991). Approximately 10 µg DNA was digested overnight with *Hind* III, *Apa* I or *Bam*HI and electrophoresed in a 1% agarose gel (Sambrook *et al.*, 1989). The DNA was blotted onto Hybond™-N+ positively charged nylon membrane using 20X SSC, in accordance with the procedure supplied by  
15 Amersham Life Science. Hybridization was conducted at 42°C in a formamide buffer using a radiolabeled probe from a 1.1 KB *hpt* (hygromycin resistance gene) fragment. The hybridized membranes were washed as recommended in the Amersham protocol and analysed using a PhosphorImager (Molecular Dynamics).

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#### *Analysis of transgenic plants by PCR*

Genomic DNA for PCR template was prepared as for Southern analysis. Oligonucleotide primers (5'-TGTGGCAGTGGAGAGAAGAG-3' (SEQ ID No 5) and 5'-ATGTTGTTGGTGATTGGTG-3' (SEQ ID No 6)) were used to identify T1  
25 progeny containing the hpBYDVpol gene. These primers amplify a 626 nt fragment from ORF2 of BYDV. The amplified region forms part of the spacer loop between sense and antisense sequences of the hpBYDVpol gene. PCR analysis was performed using AmpliTaq Gold™ according to manufacturers instructions using approximately 100 ng of genomic DNA. PCR reactions were  
30 performed in a thermal sequencer (FTS 960, Corbett research); 94°C 12 min; 5 cycles 94°C 1 min, 60°C 2 min, 72°C 3 min; 25 cycles 94°C 30 sec, 60°C 2 min,

72°C 1 min 30 sec; 70°C 1 min. Electrophoretic analysis of PCR products was conducted using 1% agarose gels buffered in 1 x TBE.

### Example 1: Construction of the hairpin gene (hpBYDVpol)

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A full-length BYDV-polymerase (BYDVpol) sequence was amplified from a cDNA clone of an Australian BYDV-PAV isolate (GenBank Accession No. D11032) using GeneAmp XL PCR kit (Perkin Elmer), with a pair of primers (5'-ACCATTCTATTGTGCTCTCGCACAGAGATAAGCAGGAAACCATGGTTTTCGA  
10 AATACTAATAGGT-3' (SEQ ID No 3) and 5'-CCGGAATTCTTAATATTCGTTTTGTGAG-3' (SEQ ID No 4) that introduced a *Nco* I site and an *Eco*R I site at the 5' end and the 3' end, respectively. The underlined nucleotides are from the BYDV-PAV sequence. The resulting PCR fragment was digested with *Nco* I and *Eco*R I, and cloned into pUJJT (Wang and  
15 Waterhouse, 2000) at the corresponding sites, forming the Ubi1-JGMV5'-BYDVpol-tm1' cassette. A 1.6 kb sequence from the 5' half of BYDVpol was excised with *Nco* I and *Bam*H I from the PCR fragment, treated with Klenow polymerase to generate blunt ends, and inserted into the *Eco*R I site (also pre-treated with Klenow) between BYDVpol and tm1' in the Ubi1-JGMV5'-BYDVpol-  
20 tm1' cassette, giving the hairpin gene hpBYDVpol (Fig. 1). For barley transformation, the hairpin gene cassette was excised with *Not* I and inserted into pWBVec8, which contains an intron-interrupted hygromycin resistance gene, as the selectable marker, in the T-DNA region (Wang et al., 1998b).

### 25 Example 2: Transformation and Analysis of T<sub>0</sub> plants

A gene construct (hpBYDVpol) was made in which a hairpin RNA, containing BYDV-PAV polymerase gene sequences, is transcribed under the control of the maize ubiquitin promoter (Fig. 1). Using this construct and an *Agrobacterium*-  
30 mediated transformation system, an overall transformation efficiency of 13% was achieved resulting in 38 independent transgenic barley plants (Table 1).

Table 1. Transformation of barley with hpBYDVpol.

Strain of <i>Agrobacterium</i>	Acetosyringone conc. in co-cultivation medium ( $\mu$ M)	No. scutella used	No. hygromycin resistant calli generated	No. transgenic lines generated
AGL0	0	72	40 (55%) <sup>A</sup>	17 (24%)
	200	70	40 (57%)	8 (11%)
AGL1	0	71	17 (24%)	6 (8%)
	200	72	22 (30%)	7 (10%)

<sup>A</sup> Numbers in brackets = No. transgenic calli or plants / No. scutella (as a percentage)

Southern analysis indicated that 19 plants carried a single transgene copy, 12 contained two copies and 7 had three or more copies. The analysis of the first 15 of these plants is shown in Figure 2. When 25 of the T<sub>0</sub> plants were inoculated with BYDV-PAV, 9 of them appeared highly resistant as they supported little or no virus replication when measured by ELISA (data not shown). Six lines (1 – 6) were selected for further analysis; four of these appeared highly resistant and two appeared susceptible to BYDV-PAV infection (Table 2).

Table 2. Testing T<sub>0</sub> and T<sub>1</sub> hpBYDVpol plants for virus resistance.

T <sub>0</sub> Line number	T <sub>0</sub> Reaction to BYDV-PAV	T <sub>0</sub> Number of transgene copies (Southern)	T <sub>1</sub> Segregation Highly resistant: Susceptible (ELISA)
1	Highly resistant	>5	1:2
2	Highly resistant	1	18:12 <sup>A</sup>
3	Susceptible	1	0:15
4	Highly resistant	1	17:10 <sup>A</sup>
5	Highly resistant	2	15:2 <sup>B</sup>
6	Susceptible	>5	0:30

<sup>A</sup> The segregation for BYDV resistance versus susceptibility conforms to a 3:1 ratio for a single dominant locus (Chi-square test,  $p > 0.05$ ).

<sup>B</sup> The segregation for BYDV resistance versus susceptibility conforms to a 15:1 ratio for two dominant loci (Chi-square test,  $p > 0.05$ ).

### Example 3: Analysis of T<sub>1</sub> plants for response to BYDV-PAV inoculation.

About 20 seed from each of the 6 T<sub>0</sub> Lines were sown in soil. The resulting plants were inoculated with BYDV-PAV and assayed by ELISA three weeks later (Table 3).



Table 3. Virus level, measured by ELISA, for groupings of highly resistant and susceptible T1 progeny of transgenic Lines 2 and 4, following inoculation with either BYDV-PAV or CYDV-RPV.

Challenge virus	BYDV-PAV		CYDV-RPV	
	Highly resistant	Susceptible	Highly resistant	Susceptible
Line 2	0.103±0.001 <sup>A</sup> (9) <sup>B</sup>	1.148±0.074 (6)	- (0)	1.136±0.061 (15)
Line 4	0.109±0.036 (9)	1.741±0.075 (3)	- (0)	1.373±0.107 (15)
Control +	1.040±0.172 (5)		1.070±0.102 (6)	
Control -	0.105±0.003 (6)		0.147±0.003 (6)	

<sup>A</sup> ELISA reading (with Standard Errors) of plants, 28 days post inoculation

<sup>B</sup> Numbers of plants are shown in brackets

Control + = challenged wildtype plants; Control - = unchallenged wildtype plants

All of the plants from Lines 3 and 6 were as susceptible to BYDV-PAV infection as their T<sub>0</sub> parents had been, whereas the progeny of each of the other lines (which were all resistant as T<sub>0</sub> plants) contained some plants that were susceptible and others that were resistant. The T<sub>0</sub> Line 1 plant produced a total of eighteen seed and of these only three germinated. While one of the three plants that grew was found to be resistant to BYDV, the other two were found to be susceptible. The progeny of Lines 2 and 4 conformed to a segregation ratio of 3:1 (highly resistant: susceptible), suggesting the presence of a single dominant transgene locus in each line and Southern analysis (Fig.3) revealed that the loci each appear to contain a single transgene. Plants from Line 5 conformed most closely to a segregation ratio of 15:1 (highly resistant: susceptible), implying the presence of two dominant loci. Taken together, the results suggested that the hpBYDVpol transgene was conferring extreme resistance to BYDV-PAV in four of the six transgenic barley lines analysed and, in three of them (Lines 2, 4 and 5), this was inherited in a predictable Mendelian manner.

*Transgene inheritance and virus immunity in hpBYDVpol Lines 2 and 4*

Inheritance of the hpBYDVpol transgene and of BYDV-PAV resistance was further examined in Lines 2 and 4. Fourteen T<sub>1</sub> plants of each line were  
5 challenged with BYDV-PAV and monitored for virus symptoms and virion accumulation 21, 28, and 42 days after inoculation. The inheritance of the hpBYDVpol transgene in these plants was examined by PCR amplification of a 626 bp fragment of DNA from the transgene. In both lines (Fig. 4), the inheritance of hpBYDVpol correlated perfectly with lack of virus symptoms and  
10 resistance to BYDV infection. Plants that were symptomless and contained undetectable virus levels 21 days after inoculation and remained this way throughout the six weeks of analysis (Fig. 5A), whereas those plants without the hpBYDVpol transgene showed virus symptoms and virion accumulation at all three time-points. The co-segregation of virus accumulation with the absence of  
15 the transgene was also evident in the grain yield from the individual progeny plants. In Line 2, the average grain yield from the nine hpBYDVpol-containing T<sub>1</sub> progeny was  $29.7 \pm 1.8$  g compared to a yield of  $20.9 \pm 1.5$  g from the six transgene-free progeny. Twenty-eight days after inoculation, the ELISA level in plant 12 of Line 4 (Fig. 4) suggested that some virus accumulation had occurred.  
20 However, the plant had undetectable levels of BYDV-PAV both one week before and two weeks after this time-point, and never developed virus symptoms. This suggests that there might have been a low level of virus replication, peaking at 28 days, which was then overcome by the transgene-induced resistance or that there was some contamination of the sample during analysis.

25

*Specificity and robustness of the virus resistance*

Although BYDV-PAV is the major virus pathogen of cereals in the field, CYDV-RPV can also be present. Indeed the two viruses can occur as a complex and appear to have a synergistic effect on symptom severity (Fig. 5B). The viruses  
30 share common hosts, a common insect vector and many aspects of their biology. However, they are at opposite ends of the luteovirus spectrum in terms of

sequence homology and genome organisation. To test whether the hpBYDVpol transgene also conferred protection against CYDV-RPV, approximately 15 T<sub>1</sub> progeny from each of hpBYDVpol Lines 2 and 4 were challenged with BYDV-PAV and another 15 of each line with CYDV-RPV. Measuring the virus accumulation in these plants by ELISA revealed that, as before, both lines segregated 3:1 for BYDV-PAV resistance. However, all of the plants inoculated with CYDV-RPV were fully susceptible to the virus (Table 3). These results indicate that the resistance conferred by the hpBYDVpol transgene is specific to BYDV-PAV. This might have been expected, as there is only 34% homology between nucleotide sequence in hpBYDVpol and the corresponding region in CYDV-RPV.

Some viruses have the capacity to enhance the replication and/or spread of co-infecting viruses and to inactivate PTGS (Vance 1991; Vance *et al.*, 1995; Pruss *et al.*, 1997; Shi *et al.*, 1997; Voinnet *et al.*, 1999). Therefore, it was possible that infection by CYDV-RPV, which can co-infect with BYDV-PAV in the field, could enhance the replication and spread of BYDV-PAV or inactivate the hpBYDVpol-mediated BYDV-PAV immunity, thus disarming the plant's newly conferred protection. It has recently been shown that some viruses have the capacity to inactivate PTGS (Voinnet *et al.* 1999). The P1 protein of the Sobemovirus, *Rice yellow mottle virus*, inactivates PTGS (Voinnet *et al.*, 1999). Its gene may be orthologous to ORF1 (and possibly parts of ORF2) of CYDV-RPV.

Therefore, it was important to determine whether infection by CYDV-RPV could inactivate the hpBYDVpol-mediated BYDV-PAV resistance. To test this, 15 T<sub>1</sub> progeny from hpBYDVpol Line 4 were inoculated with the B/CYDV-MIX isolate, a virus complex containing BYDV-PAV and CYDV-RPV. The plants were subsequently tested using species-specific ELISA for accumulation of BYDV-PAV and CYDV-RPV and by PCR for inheritance of the transgene. The results (Fig. 6) showed that CYDV-RPV replicated to high levels in all 15 plants but that the 12 plants inheriting the hpBYDVpol transgene were resistant to BYDV-PAV.

This shows that the resistance to PAV is not compromised by replication of CYDV-RPV and further confirms the 3:1 (BYDV-PAV resistance: susceptible) segregation ratio.

5 *Recovery of virus from virus-challenged hpBYDVpol plants*

Although BYDV-PAV-challenged hpBYDVpol-plants contain extremely low levels of BYDV-PAV antigen, they might contain sufficient virus to be acquired by aphids and thus be of ecological significance. To examine this, we attempted to recover infectious virus from T1 progeny plants from lines 2 and 4 that had been  
10 previously challenged with either BYDV-PAV or B/CYDV-MIX. Virus-free aphids were fed (for three days) on the plants ten weeks after the initial challenge and then transferred to healthy test plants (Table 4).

Table 4. Recovery of virus from transgenic and non-transgenic barley plants inoculated with BYDV-PAV or B/CYDV-MIX.

Challenge virus	Genotype <sup>A</sup>	Recovery of BYDV-PAV <sup>B</sup>	Recovery of CYDV-RPV <sup>B</sup>
BYDV-PAV	Line 2 + t	0/4 (0.084 ± 0.004) <sup>C</sup>	-
	Line 2 - t	1/1 (2.069)	-
	Line 4 + t	0/4 (0.076 ± 0.003)	-
	Line 4 - t	1/1 (1.185)	-
	Control	3/3 (1.212 ± 0.081)	-
B/CYDV-MIX (BYDV-PAV and CYDV-RPV)	Line 2 + t	0/4 (0.069 ± 0.004)	4/4 (0.895 ± 0.174)
	Line 2 - t	1/1 (1.191)	1/1 (0.819)
	Line 4 + t	0/4 (0.094 ± 0.025)	4/4 (1.576 ± 0.134)
	Line 4 - t	1/1 (0.954)	1/1 (0.909)
	Control	3/3 (1.108 ± 0.166)	3/3 (1.473 ± 0.300)

<sup>A</sup> + t = segregant containing the hpBYDVpol transgene; - t = segregant without hpBYDVpol

<sup>B</sup> Number of test plants infected / Number inoculated

<sup>C</sup> Numbers in brackets are average ELISA readings and associated Standard Errors

Whereas the test plants became infected with BYDV-PAV from aphids fed on BYDV-PAV or B/CYDV-MIX challenged wildtype or non-transgene segregant plants, none of them was infected with BYDV-PAV from aphids fed on similarly challenged plants containing the hpBYDV-PAVpol transgene. However, aphids did recover CYDV-RPV from hpBYDVpol plants challenged with the B/CYDV-MIX mixture. Taken altogether, the data show that BYDV-PAV-challenged hpBYDVpol

plants contain no biologically active virus and should be regarded as immune to BYDV-PAV.

**Example 4: Construction of a DNA construct encoding dsRNA with multiple  
5 target regions in the BYDV and CYDV genomes.**

Using conventional recombinant DNA techniques, the following chimeric genes are constructed comprising the following operably linked DNA elements:

- 10 • a maize ubiquitin promoter region (Christensen and Quail, 1996) or subterranean clover stunt virus promoter S4 region or subterranean clover stunt virus promoter S7 region comprising an Adh1 intron (WO 9606932)
- a first nucleotide sequence comprising in order
  - 15 a. the nucleotide sequence of SEQ ID No 7 from nucleotide 2314 to nucleotide 2514;
  - b. the nucleotide sequence of SEQ ID No 7 from nucleotide 429 to nucleotide 629;
  - c. the nucleotide sequence of SEQ ID No 7 from nucleotide 5052 to nucleotide 5270;
  - 20 d. the nucleotide sequence of SEQ ID No 8;
  - e. the nucleotide sequence of SEQ ID No 9;
- a second nucleotide sequence which comprise the complement of the first nucleotide sequence
- a transcription termination and polyadenylation signal

25

The DNA molecule is introduced into barley plants, and BYDV/CYDV resistant barley plant lines are isolated.

30



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We claim

- 1) A DNA molecule comprising
  - a) a plant-expressible promoter;
  - 5 b) a DNA region which when transcribed in the cells of a cereal plant yields a RNA molecule comprising
    - i) a first nucleotide sequence of at least 19bp having at least 70% nucleotide sequence identity to the sense nucleotide sequence of a BYDV isolate encoding RNA dependent RNA polymerase; and
    - 10 ii) a second nucleotide sequence of at least 19bp having at least 70% nucleotide sequence identity to the complement of said sense nucleotide sequence of a BYDV isolate encoding RNA dependent RNA polymerase; and
    - iii) a transcription termination and polyadenylation signal;
- 15 wherein said first and second nucleotide sequences are capable of forming a double stranded RNA by base pairing between regions which are complementary.
- 2) The DNA molecule of claim 1, wherein said nucleotide sequence encoding  
20 said polymerase comprises the nucleotide sequence of SEQ ID 1 from the nucleotide at position 1 to the nucleotide at position 1014.
- 3) The DNA molecule of claim 1, wherein said first nucleotide sequence  
25 comprises the nucleotide sequence of SEQ ID No 1 and said second nucleotide sequence comprises the complement of the nucleotide sequence of SEQ ID No 1.
- 4) The DNA molecule of claim 1, wherein said DNA region contains a nucleotide  
30 sequence encoding a spacer region between a DNA region encoding said first nucleotide sequence and a DNA region encoding said second nucleotide sequence.



- 5) The DNA molecule of claim 3, wherein said DNA region contains a nucleotide sequence encoding a spacer region between a DNA region encoding said first nucleotide sequence and a DNA region encoding said second nucleotide sequence and wherein said DNA region encoding said spacer region has the nucleotide sequence of SEQ ID No 2.
- 6) A DNA molecule comprising
- a) a plant-expressible promoter;
  - b) a DNA region which when transcribed in the cells of a cereal plant yields an RNA molecule comprising
    - i) a first nucleotide sequence of at least 19bp having at least 70% nucleotide sequence identity to the sense nucleotide sequence of a BYDV isolate encoding RNA dependent RNA polymerase, further comprising at least one and maximally 10 additional nucleotide sequences, each of said additional nucleotide sequence being at least 19bp in length and having at least 70% nucleotide sequence identity to a part of the sense nucleotide sequence of a BYDV isolate genomic sequence or a CYDV isolate genomic sequence ; and
    - ii) a second nucleotide sequence complementary to said first nucleotide sequence; and
    - iii) a transcription termination and polyadenylation signal;
- wherein said first and second nucleotide sequences are capable of forming a double stranded RNA by base pairing between regions which are complementary.
- 7) A DNA molecule according to claim 6 wherein said first nucleotide sequence comprises in order the following operably linked elements:
- a. a nucleic acid comprising the nucleotide sequence of SEQ ID No 7 from the nucleotide at position 2314 to the nucleotide at position 2514;

- b. a nucleic acid comprising the nucleotide sequence of SEQ ID No 7 from the nucleotide at position 429 to the nucleotide at position 629;
  - c. a nucleic acid comprising the nucleotide sequence of SEQ ID No 7 from the nucleotide at position 5052 to the nucleotide at position 5270;
  - d. a nucleic acid comprising the nucleotide sequence of SEQ ID No 8; and
  - e. a nucleic acid comprising the nucleotide sequence of SEQ ID No 9.
- 8) A method for producing a cereal plant resistant to a Barley Yellow Dwarf virus comprising the steps of
- a) producing a population of transgenic cereal plant lines comprising the DNA molecule of any one of claims 1 to 7 integrated into the genome of the cells of transgenic plant of said plant line;
  - b) isolating a transgenic cereal plant line resistant to Barley Yellow Dwarf virus infection.
- 9) The method of claim 8, wherein said cereal plant is selected from the group of wheat, barley and oats.
- 10) The method of claim 8, wherein said cereal plant is barley.
- 11) The method of claim 8, further comprising the step of c) crossing said isolated transgenic cereal plant line resistant to Barley Yellow Dwarf virus infection to another cereal plant to obtain progeny plants comprising the DNA molecule of any one of claims 1 to 7.
- 12) A method for producing a cereal plant resistant to a Barley Yellow Dwarf virus in the presence of a PTGS inactivating protein comprising the step of

- a) producing a population of transgenic cereal plant lines comprising the DNA molecule of any one of claims 1 to 7 integrated into the genome of the cells of transgenic plant of said plant line;
- b) isolating a transgenic cereal plant line resistant to Barley Yellow Dwarf virus infection.
- 5
- 13)The method of claim 12, wherein said PTGS inactivating protein is encoded by a co-infecting virus.
- 10
- 14)The method of claim 13, wherein said co-infecting virus is Cereal Yellow Dwarf virus.
- 15)Use of a DNA molecule of any one of claims 1 to 7 to produce a transgenic cereal plant resistant to BYDV.
- 15
- 16)Use of a DNA molecule of any one of claims 1 to 7 to produce a transgenic cereal plant resistant to BYDV in the presence of Cereal Yellow Dwarf virus.
- 17)A cereal plant comprising stably integrated into the genome of the cells of said plant the DNA molecule according to any one of claims 1 to 7 and wherein said cereal plant is resistant to BYDV virus infection and replication of said virus.
- 20
- 18)The cereal plant of claim 17, which is selected from the group consisting of wheat, barley, rye and oat.
- 25
- 19)The cereal plant of claim 17, which is barley.

Fig. 1

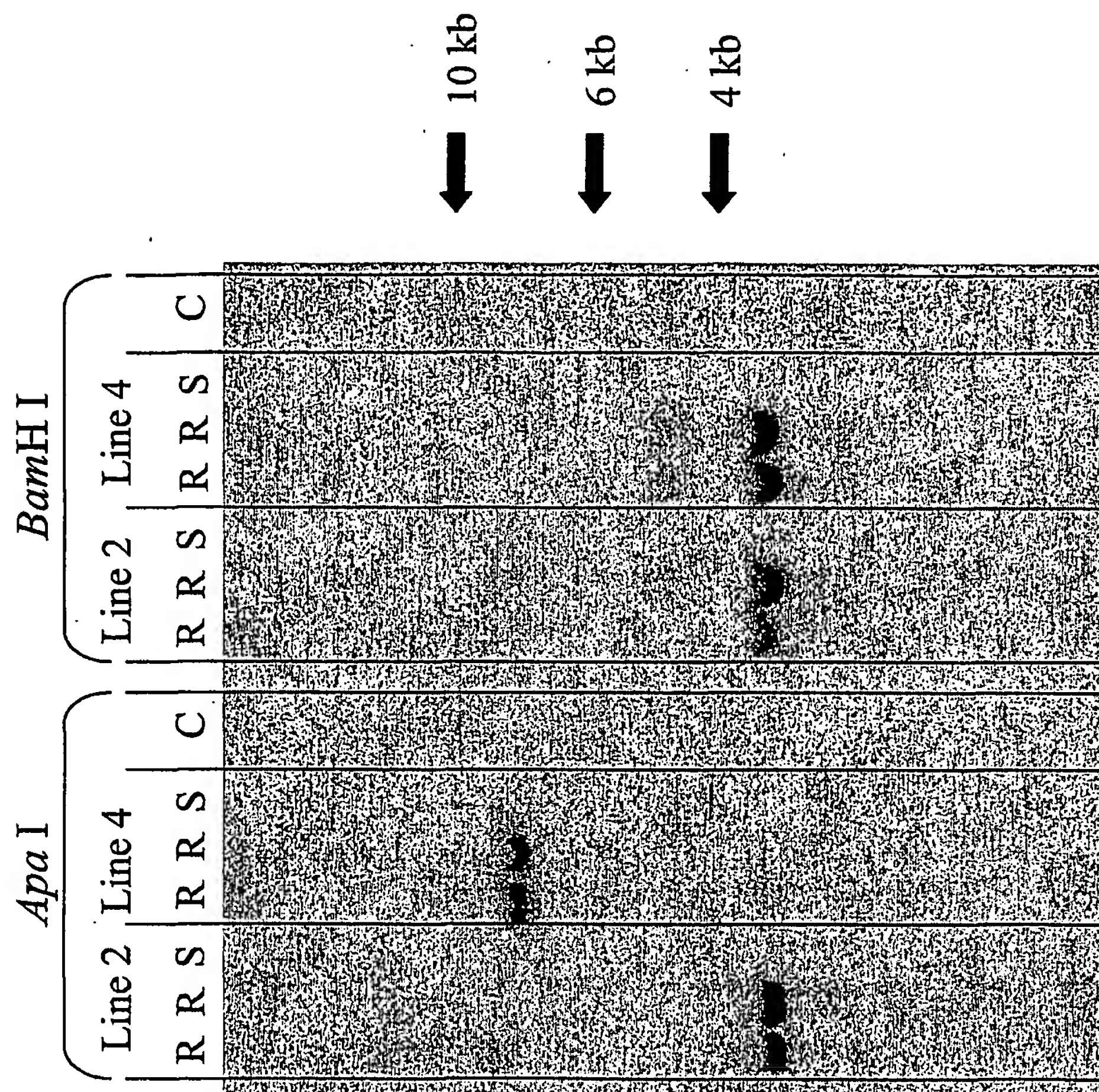
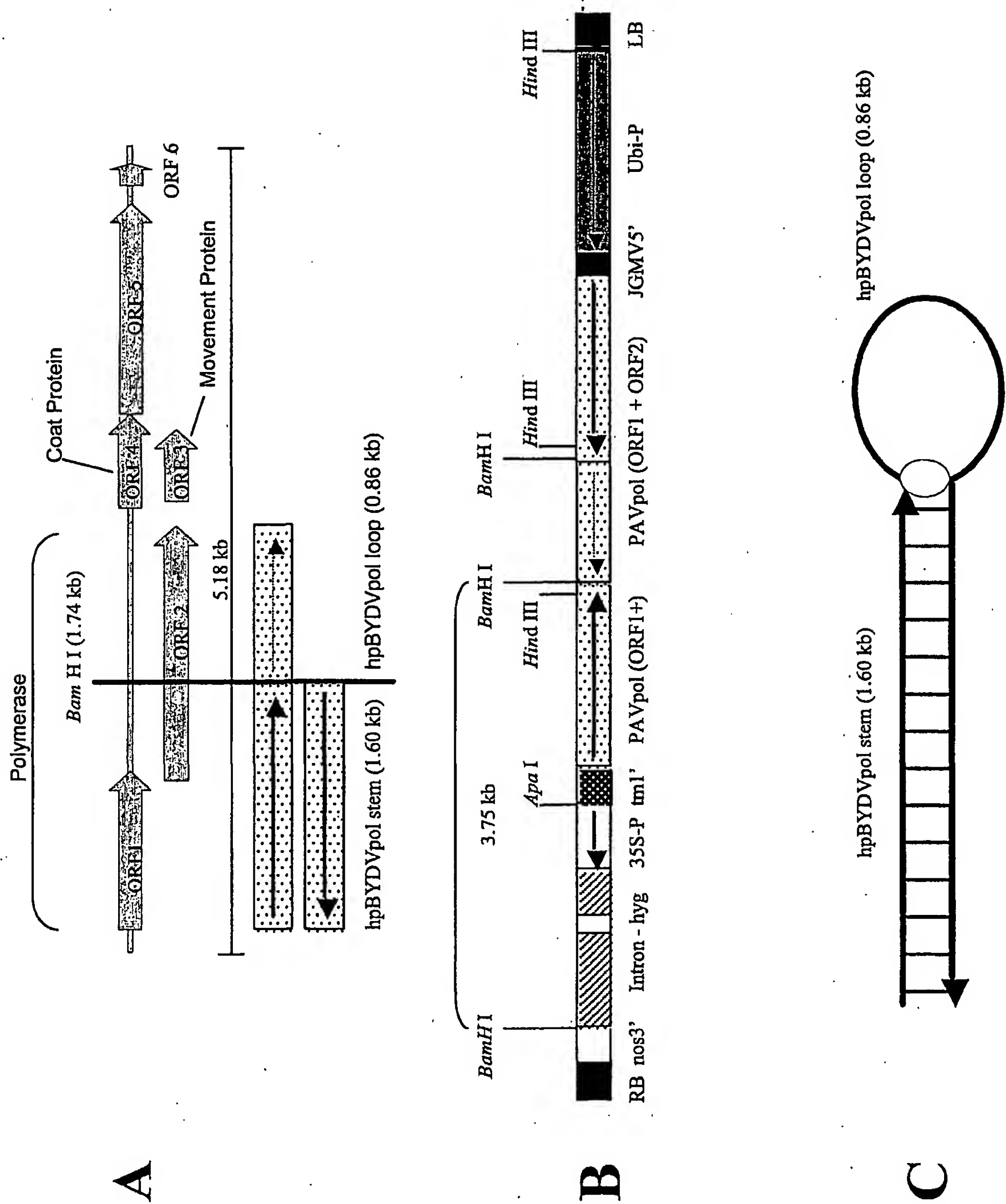


Fig. 2



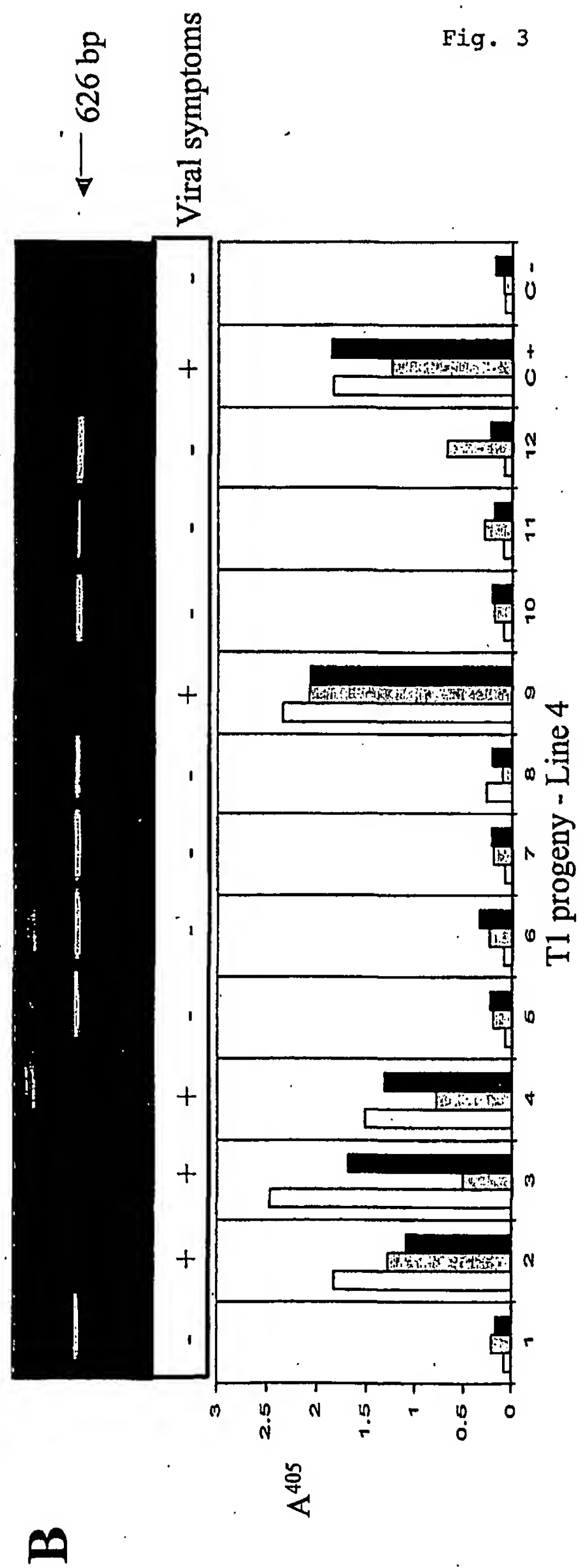
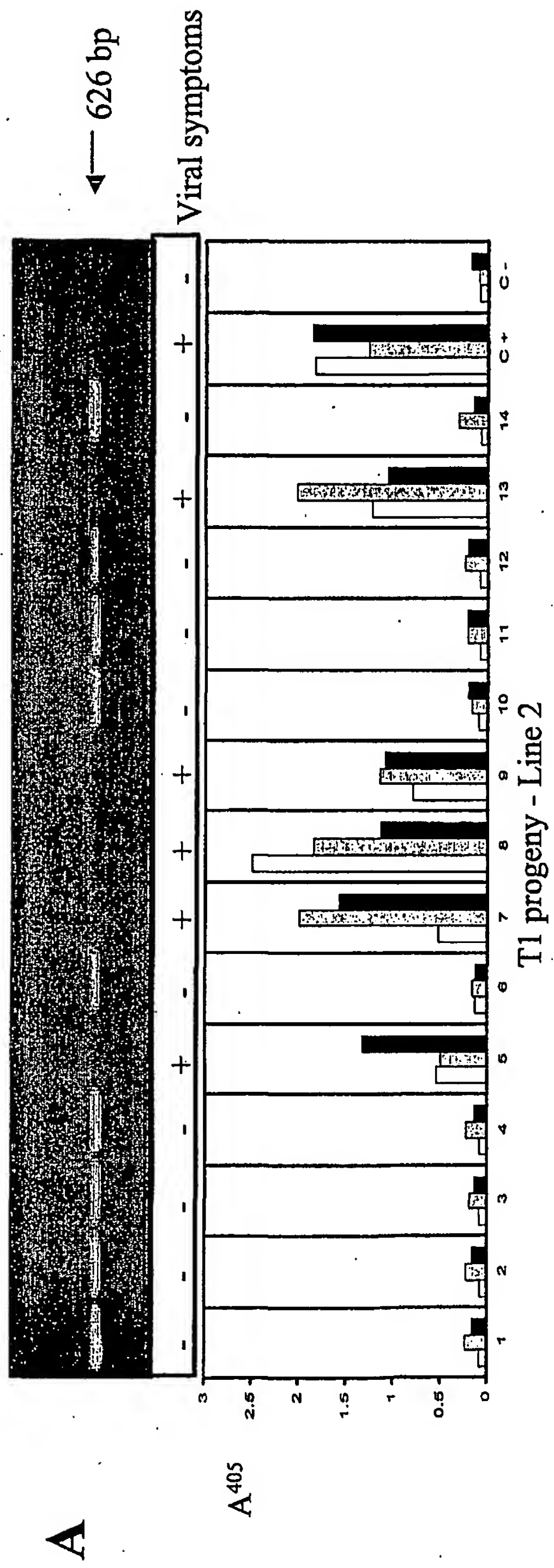


Fig. 3



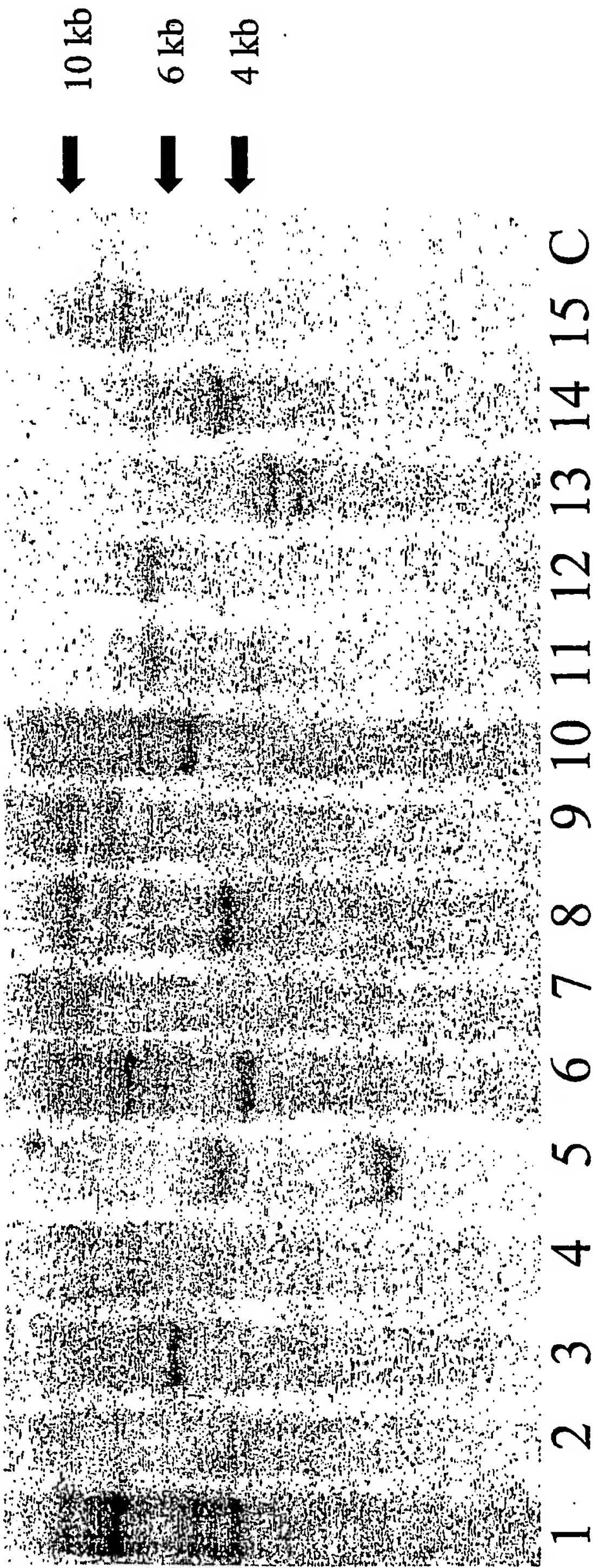


Fig. 4

Fig. 5

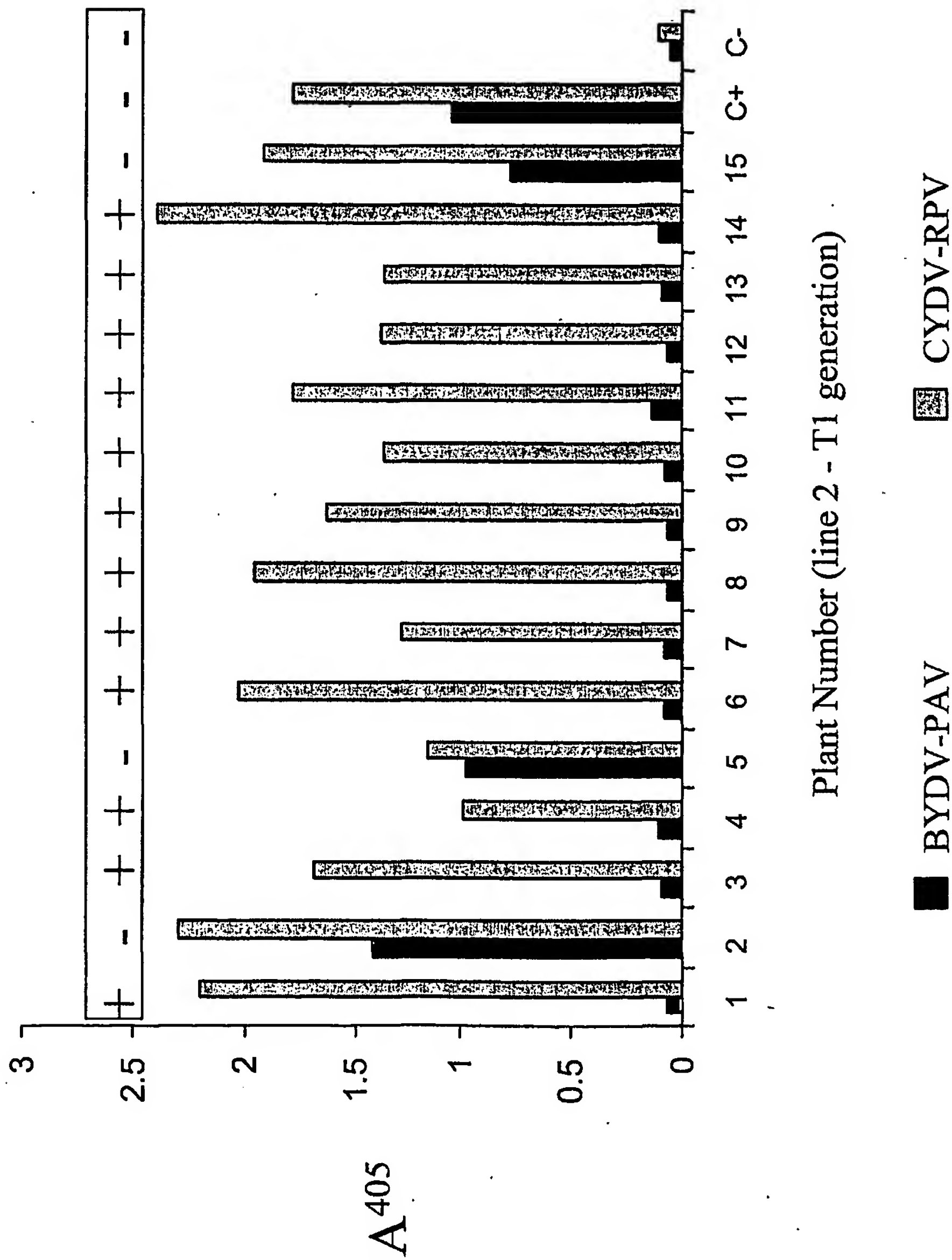
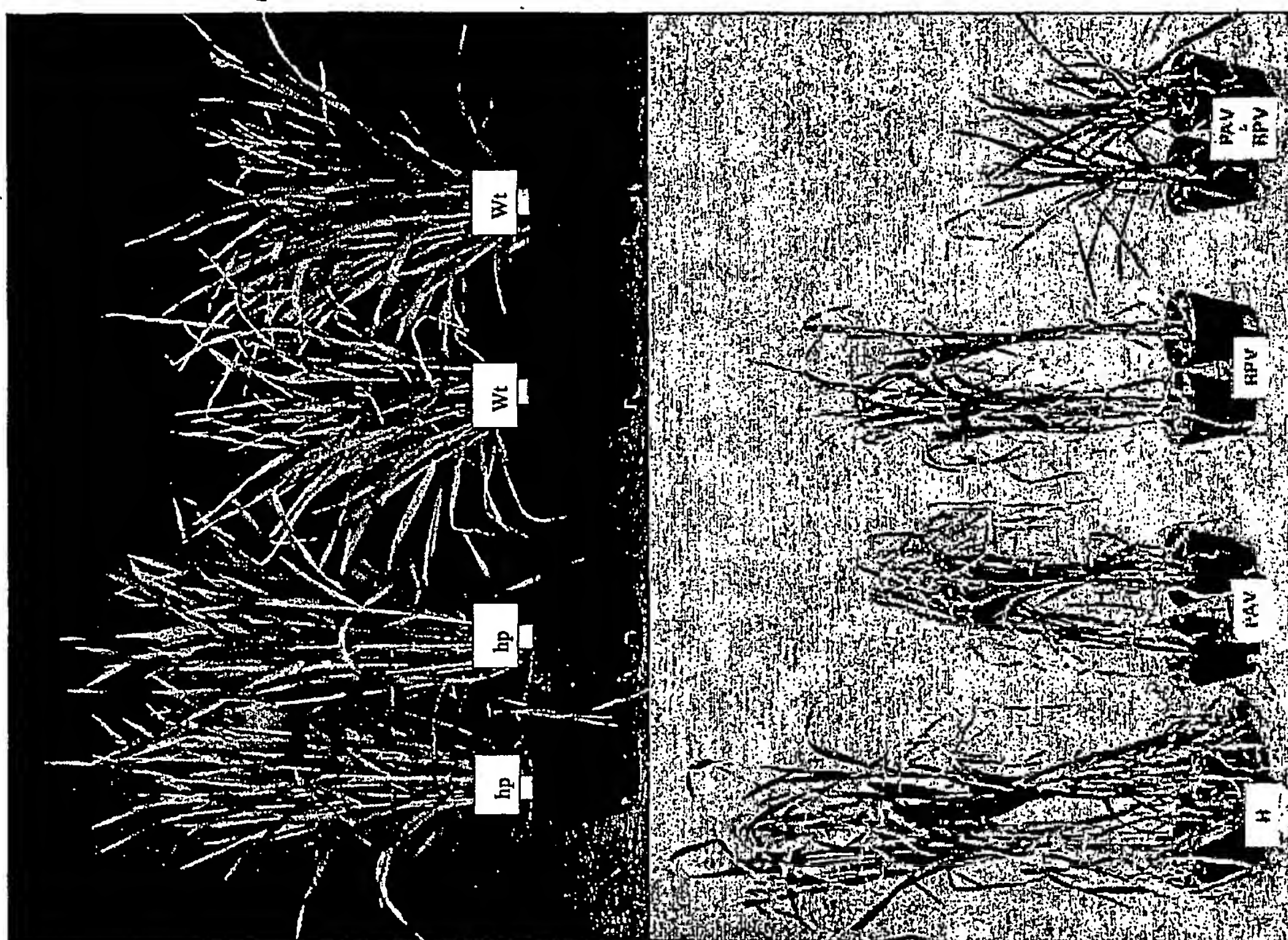


Fig. 6



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